Master's Thesis

Engineering *Escherichia coli* Membrane Vesicles to Transfect Mammalian Cells *in Vitro*

SooYeon Kim

Department of Biological Sciences

Graduate School of UNIST

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SooYeon Kim

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Advisor
Robert J. Mitchell

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This certifies that the thesis/dissertation of SooYeon Kim is approved.

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Advisor: Robert J. Mitchell

Prof. Sung Kuk Lee

Prof. MyungGon Ko

Abstract

Membrane vesicles (MVs) are the nano-sized structures membrane lipid formed from budding off of the outermost membrane of a cell. MVs are known to package a variety of cargo, including small molecules, peptides, proteins and genetic material. Based on their assorted cargo, MVs are known to transport DNA, RNA, and Proteins across the extracellular space. The unique characteristics could be utilized for therapeutic applications.

In this study, *Escherichia coli (E. coli)* BW25113 strain will be engineered to mass produce DNA containing MVs by knocking out the NlpI gene and transforming with Red Fluorescent Protein(mCherry). Also, INV (gene for invasion)/LLO (gene synthesizing listeriolysin O) plasmid will be transformed into the *E. coli* strains for active transport of the MVs into the target mammalian cell. The characteristic of these vesicles will be analyzed using Qubit 3 fluorometer system to measure the concentration of the MVs produced and DNAs in the vesicle. We will also analyze the transfect ability of the MVs produced to the mammalian cell lines such as Hela, the human cervical cancer cell, and HEK293, the human embryonic kidney cell, which are good models for transfection. The toxicity and the immunogenicity of invasive vesicles will be characterized using MTT assay and Enzyme-Linked Immunosorbent Assay(ELISA). Throughout the study, we will demonstrate the MV as a transfecting agent.

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Abbreviation

MV : Membrane vesicle

E. coli : *Escherichia coli*

DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ELISA : Enzyme-Linked Immunosorbent Assay

USA : United States of America

DMSO : Dimethyl sulfoxide

MEM : Minimum Essential Media

DMEM : Dulbecco's Modified Eagle Media

DPBS : Dulbecco's Phosphate-Buffered Saline

EDTA : Ethylenediaminetetraacetic acid

RPM : Revolution per minute

RCF : Relative Centrifugal Force

TE : Trypsin-EDTA

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CFU : Colony forming unit

LB : Luria-Bertani broth

OD : Optical density

RFP : Red Fluorescent Protein

w.t. : wild type

PG : Peptidoglycan

Chapter 1 Introduction

1.1 Transfection of mammalian cell

As a modern biological understanding and technology improve, the needs for patient-specific treatment increases.[1] The genetic manipulation is a key technique for both enriching the biological knowledge and developing individualized gene therapy.[2] The transfection is the core technology used in the laboratory to reveal the function of the specific gene or protein of interest.[3, 4] Thus varieties of transfection methods have been developed.[5, 6] The methods can be categorized into three different departments; a primarily developed biological method, a chemical method that is widely used in laboratory and easy to use, and Physical method.

In biological transfection, a viral vector was first developed to transduce the DNA of interest into a human cell. By utilizing the viral vector, the gene of interest can be inserted into the expression vector and virus can transfer the plasmid into the target cell.[7] The virus can be applied to *in vivo* with high expression rate once transfected due to viral expression vector has viral promotor which is overexpressed in target mammalian cell. [8] Despite its advantages, the viral vector is difficult to mass produce since the virus requires a host in order to grow. Likewise, the generation and titration of the vector are cumbersome. The viral vector can possibly generate unexpected mutation or induce immune response due to its viral structure.[9-12]

Chemical method is generally used to transfect the mammalian cells. Chemicals such as calcium phosphate, cationic polymers, and cationic lipid from liposomes bind to the backbone of the DNA which has a negative charge due to the phosphate group and delivers to the target cell. Although it is only applicable *in vitro*, chemical transfecting agents have high transfection efficiency and reproducibility and is easy to use, therefore has been commercialized and widely used in the laboratory.[13-16]

The physical method includes electroporation, laserfection, optoinjection, and direct injection using gene gun. Each method is applicable in specific conditions that electroporation results in high cell death so that it is well used for bacterial cells but seldom used for mammalian cells, or that laserfection and optoinjection yields are high, however, only applicable *in vitro* and requires sophisticated instruments.[17-20] The gene gun is simple and rapidly transfect; just aim and shot, without any treatment or duration, with limited application *in vivo*; muscle and tumor region, however, it is quite expensive for low transfection efficiency.[21] is summarized in Table 1.1.1

Reviewing the advantages and limitations of current transfection methods (Table 1.1.1) revealed that there is no sufficient method that can be both applicable *in vivo* and *in vitro* with high

transfection efficiency and less immunogenicity and cytotoxicity. Therefore, I suggest bacterial membrane vesicle as a DNA vector. Recently, bacterial membrane vesicles are gaining much importance because their role in pathogenesis can actually be beneficial.[22]

Table 1.1.1 Transfection Current Method Summary[23]

Technique	Delivery Mode	Advantage	Disadvantage
Biological: Viral Virus	Retrovirus Adeno-associated virus Lentivirus	Compatible for *in vivo* delivery Stable expression	Generation and titration of virus particles Difficult to produce in large quantity Possible mutagenic and immunogenic effects
Chemical	Cationic liposomes Polymer nanoparticles Lipid conjugation	High efficiency and reproducibility Easy Commercialized	Low *in vivo* efficiency
Physical: Electroporation	Voltage pulse	Delivery into any cells	High cell death Difficult to apply *in vivo*
Physical: magnetic nanoparticle	Magnetic nanoparticles	Increased transfection	Requires adherent cell
Physical: Gene gun	Heavy metal particles	Applicable to muscles and tumor Simple and rapid	Lower efficiency High cost
Physical: Laserfection/ Optoinjection	Laser light	High transfection efficiency	Expensive laser microscope system

1.2 Bacterial Membrane Vesicles

Membrane vesicles (MVs) are the nano-sized structures secreted by bacteria[24] and are produced to deliver the intracellular contents to the extracellular space. Eukaryote cells and Bacterial cells produce different kinds of membrane vesicles, for example, Gram-negative bacteria produce Outer membrane vesicles. MVs are known to package a variety of cargo, including small molecules, peptides, proteins and genetic material.[25] Based on their assorted cargo, MVs have been implicated in many biological processes ranging from cell-cell communication to gene transfer and the delivery of virulence factors, all of which depend on the bacterium producing the MVs. Outer membrane vesicles from Gram-negative bacteria can be involved in cell to cell signaling and communication and be mediators of immune regulation and pathogenesis. By virtue of their small size, their demonstrated ability to transport proteins, and their capacity to associate with mammalian cells in a receptor-dependent manner,[26] MVs present themselves as potential biotechnological tools for use in medicine and research. Moreover, recent developments in molecular biology techniques have led to engineered MVs and their application in some very exciting and unique applications where traditional nanoparticles are proving too difficult to employ.[27]

1.2.1 Outer Membrane Vesicle as a Vehicle to Deliver the Macromolecules in high concentration

On membrane vesicle formation, the membrane proteins and lipids selectively concentrate certain macromolecules on the site of the vesicle formation so that vesicle achieves a higher concentration of certain macromolecule than the concentration in the bacterial cytoplasm.[25, 28] The membrane vesicle provides protection from protease as well as DNase so that the protein or DNA cargo inside the vesicle is safely delivered to the target cell.[25]

1.2.2 MVs Deliver cargo across the Kingdom

Membrane vesicles deliver macromolecules not only between Bacteria[29] or Eukaryotic[30], but also between Bacteria and eukaryote.[31] By virtue of its small size, an ability to transport proteins and largely sized genes, and capacity to associate with mammalian cells in a receptor-dependent manner, MV presents itself as a potential biotechnological tool to be used in the medical development and biological research.[32] Moreover, recent developments in molecular biology techniques have enabled massive production of MVs from *Escherichia coli*[33] and have led to engineered MVs and their application in emerging medical area of biosimilar and drug delivery system[31] where traditional methods are proving to be too difficult to employ due to several obstacles in mass production as well as in application to varying conditions and needs.[10-12, 14-21]

1.3 Engineering Bacteria to Produce Membrane Vesicles for gene delivery

The wild-type strain of *E. coli* BW25113 does not produce enough amount of vesicles. In order to utilize the model bacteria, *Escherichia coli,* the type strain should be genetically modified to increase the production of vesicles and the transfection efficiency. The gene that I want to transfect also should be incorporated into the vesicle producer. There are several genes that are known to increase invasion of the bacteria and production of the vesicle. Invasin from *Yersinia pseudotuberculosis* and Listeriolysin from *Listeria monocytogenes* can enhance the bacterial invasion compared to non-recombinants[34-36]. The scheme of genetic engineering of MV producing *E. coli* BW25113 strain is shown (Figure 1.3.1)

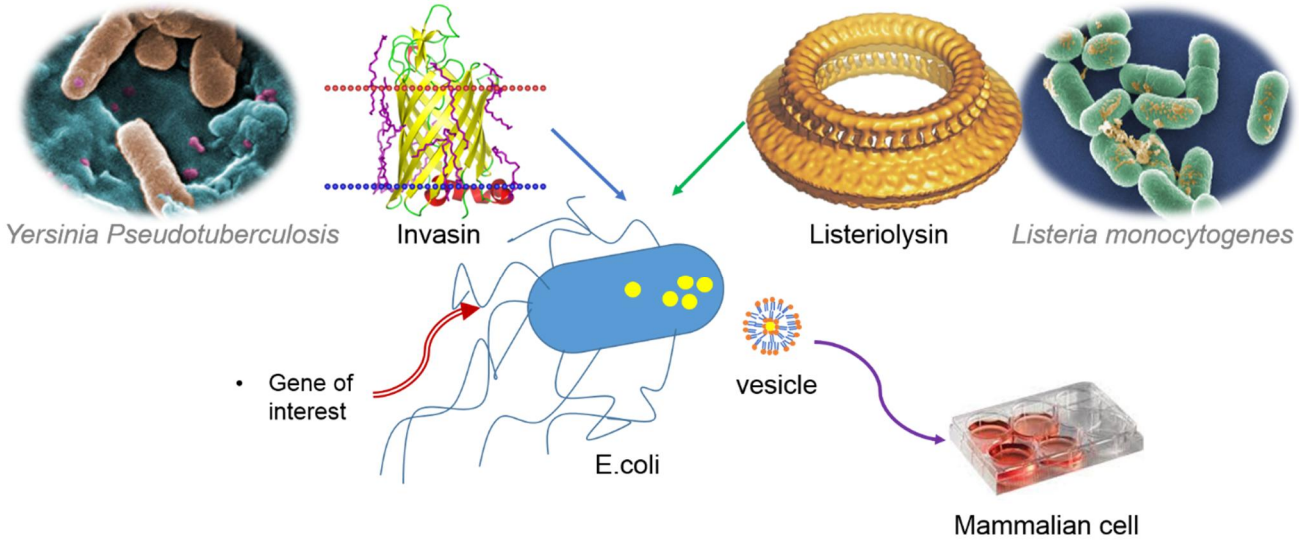

Figure 1.3.1 Scheme of *E. coli* Genetic Modification to produce invasive MVs

1.3.1 Invasin and Listeriolysin: Promotes Invasion into the Mammalian cell

Invasin is a virulence factor from *Yersinia pseudotuberculosis* that increases invasion rate when expressed in *E. coli* compared to non-recombinants. Invasin regulates adhesion and entry into the target mammalian cell through β-integrin.[35, 36]

Listeriolysin O(LLO) is a virulence factor from *Listeria monocytogenes* that allows bacteria to escape from vacuoles formed when bacteria invade into the target mammalian cell and to enter into the cytosol of the target cell. LLO can lyse the phagocytic endosome which will also increase the transfection efficiency.[34, 37]

Incorporating both INV plasmid which codes the invasion[35] and LLO plasmid which codes listeriolysin O, the recombinant bacteria produces invasin and listeriolysin O which localizes to the outer membranes of the bacteria[34]. The recombinant bacteria produce vesicles with the increased invasion to the target mammalian cell.

1.3.2 NlpI knock-out Overproduces Membrane Vesicle

E. coli itself cannot produce sufficient amount of vesicle. In order to mass produce the vesicles, the process of the biogenesis of the vesicle should be considered. Vesicles form as the outer membrane of the bacteria bud off.[22] The budding of the outer membrane occurs in the area that lacks the outer membrane-peptidoglican linking proteins. Thus, by disrupting the peptidoglycan layer, the vesiculation can be enhanced. In order to increase the vesicle production, candidate genes for vesiculation such as NlpI deletion have been considered. NlpI is a negative regulator of the peptidoglycan(PG) hydrolases. The deletion of *nlpI* results in instability of outer membrane due to PG dynamics which increases the vesiculation rate.[33, 38, 39] Therefore, *nlpI* deletion mutants which are known to show hyper-vesiculation was used in this study.

In this study, the potential of MV to be developed as a transfecting agent are demonstrated with *Escherichia coli* which are engineered to overproduce MVs by deletion of the *nlpI* gene. The engineered *E. coli* BW25113 NlpI knock-out strains yield about 100-fold higher production of MVs than *E. coli* BW25113 wild-type. The mass produced MVs are loaded with the fluorescence gene such as mCherry having mammalian promotor so that when the MVs, loaded with the dsDNA plasmid, are treated to the mammalian cell, the mammalian cell will be transfected with the fluorescence gene. Having fluorescence as an indicator of the successful transfection, the transfection using MVs was analyzed. The cytotoxicity and immunogenicity of the MVs to the transfected mammalian cell lines have been measured using the MTT assay, live-dead staining, and ELISA.

Throughout the study, we have demonstrated the potential development of the MVs as an efficient transfecting agent. Using MV as a transfecting agent instead of chemical transfection would pose a variety of advantages from low toxicity to *in vivo* applications as well as targeted transfection by

engineering MVs to have specific antigen to bind to a specific target cell. Since the genetic therapeutics is emerging strategy in personalized medication, such aspects will be beneficial.

Chapter 2 Experimental Method & Materials

2.1 Bacterial strain and growth conditions

Escherichia coli BW25113 was obtained from Keio collections. The vesicle overproducing strains were obtained by deletion of the *nlpI* gene that was done by Dr. Monnappa and Mr.Son. *E. coli* BW25113 Δ*nlpI*, *E. coli* BW25113 Δ*nlpI* INV/LLO, *E. coli* BW25113 Δ*nlpI* pCMV mCherry, *E. coli* BW25113 Δ*nlpI* pCMV mCherry INV/LLO were all obtained by transformation of each plasmid. Strains were maintained in 25% glycerol stock at -80°C. Strains of need were streaked on Luria-Bertani broth agar plates (BD Difco™, USA) with proper antibiotics for each plasmid transformed into the bacteria and grown overnight at 37°C. The bacteria with pCMV mCherry was grown with 50μg/ml of kanamycin, and the one with INV/LLO gene plasmid was grown with 35μg/ml of chloramphenicol, and the bacteria with Lux gene plasmid was grown with 100μg/ml of ampicillin. From the agar plates, a single colony was taken by the disposable loop and inoculated into LB broth and grown at 37°C overnight with agitation (250rpm) in shaking incubation.

2.2 Culture of human cervical cancer cell line (Hela cells)

Human Cervical cancer cells (Hela cells) were stocked in 5% DMSO with the complete media in 2ml cryo-tube and stored in liquid nitrogen (-200°C). Upon demand, one stock, total of 1ml, were taken out to be plated on the T25 flask using whole stock with 5ml of MEM (1X) [+] Earle's Salts, [+] L-Glutamine media (Gibco®, USA) with 10% FBS and antibiotic Normocin (InvivoGen, USA) 100μg/ml. After overnight growth in the 5% CO_2 incubator at 37°C, the media was changed to remove the remaining DMSO. Removal and handling with the mammalian cell media are done with a serological pipet(SPL LIFE SCIENCES, Republic of Korea). When the Hela cells become confluent, media were removed and cells were washed with sterile DPBS and trypsinized using Trypsin-EDTA(TE) for 5 minutes at 37°C. The detached cells were washed with 10ml of complete media (MEM + 10% FBS + 100μg/ml Normocin) and collected in 50ml tube, then centrifuged for 5 minute, 1500rpm(5600rcf). The supernatants were discarded to remove TE and 10ml of fresh complete media were added to the cell pellets and resuspended. the proper volume of cells, depending on cell concentration, ranging from 0.5ml to 2ml were added to 15ml of complete media in the T75 flask (75cm^2, Filter Cap, SPL LIFE SCIENCES, Republic of Korea). After culturing cells until they are confluent, the same trypsinization steps were processed. The confluency of the cell was observed on OLYMPUS CKX31(OLYMPUS Corporation, Japan). Using hemocytometer (iNCYTO, DHC-N01-5), cells were counted and diluted appropriately to be plated in an appropriate culture dish for the experiment.

2.3 Culture of human embryonic kidney cell line (HEK293 cells)

Embryonic kidney cells (HEK293 cells) were stocked in 5% DMSO with the complete media and stored in liquid nitrogen (-200°C). Upon demand, one stock, total of 1ml, were taken out to be plated on the T25 flask using whole stock with 5ml of DMEM-F12 media with sodium carboxide (Gibco®, USA), 10% FBS and antibiotic Normocin (InvivoGen, USA) 100μg/ml. After overnight growth in the 5% CO_2 incubator at 37°C, the media was changed to remove the remaining DMSO. When the HEK293 cells become confluent, media were removed and cells were washed with sterile DPBS and trypsinized using Trypsin-EDTA(TE) for 5 minutes at 37°C. The detached cells were washed with 10ml of complete media (DMEM + 10% FBS + 100μg/ml Normocin) and collected in 50ml tube, then centrifuged for 5 minute, 1500rpm(5600rcf). The supernatants were discarded to remove TE and 10ml of fresh complete media were added to the cell pellets and resuspended. the proper volume of cells, depending on cell concentration, ranging from 0.5ml to 2ml were added to 15ml of complete media in the T75 flask. After culturing cells until they are confluent, the same trypsinization steps were processed. Using hemocytometer, cells were counted and diluted appropriately to be plated in an appropriate culture dish for the experiment.

2.4 Transformation of the bacterial strain

The cell of interest was streaked onto the LB agar(1.6~1.7%) plate with proper antibiotics and incubated at 37°C for overnight. A single colony was taken for inoculation in 5ml of LB broth in a 15ml tube(Falcon) and incubated at 37°C shaking incubator. The inoculants were taken to be diluted 100 folds according to OD_{600} 1.0 in 5ml LB in a 15ml tube. The diluents were incubated until the OD_{600} 0.3 for 3 to 4 hours at 37°C shaking incubator. 1ml of inoculum were centrifuged in the 1.5ml tube(SPL LIFE SCIENCES, Republic of Korea) for 1 minute at max speed. Supernatants were removed and cell pellets were washed with ice-cold(4°C) 10% glycerol 500μl, 250μl, 100μl, each time centrifuging for 1min, max speed, at 4°C. Washed cell pellets were resuspended and mixed with 2ng of the plasmid. 20μl of the mixture was placed in ice-cold electrophoresis cuvette (BIO-RAD, Gene Pulser® Cuvette) and inserted into the electroporator(Eppendorf, Electroporator 2510). After applying 1800V current, the mixture was resuspended in 200μl of LB media without antibiotics and incubated at 37°C for 30 minutes to 1 hour. The samples were plated on proper LB agar plate and incubated at 37°C overnight. Some of the colony (10 to 20 colony) are selected and patched on new plate, them the plasmid from the colony were purified using plasmid & DNA purification kit (COSMO GENETECH, Republic of Korea) and the size of the plasmid was determined using electroporation with 1% agar gel (Molecular Biology

Grade, PhileKorea TECH INC, Republic of Korea) and checked with Molecular Imager® Gel Doc™ XR+(BIO-RAD, USA) for confirmation. Recombinant strains were inoculated in 5ml LB overnight and stored in 25% glycerol at -80°C.

2.5 Membrane vesicle isolation

Vesicle producing strains were streaked on proper LB agar plate and incubated at 37°C overnight. A single colony was taken for inoculation in 5ml LB broth in a 15ml tube. After inoculation for overnight, the inoculum was diluted into 1000 fold of OD_{600} 1.0 in 500ml LB broth in the 1L conical flask. The flask was incubated at 37°C shaking incubator for 24 hours. The cells were centrifuged at 7000 RPM for 15 minutes then filtered through a 0.22μ filter to remove all the cells and cell debris. The vesicles in the filtrate were concentrated using Amicon 100K centrifugal filter on 5,000 RPM(2935 X g RCF) at room temperature(22 to 24°C) using Centrifuge 5430R(Eppendorf, USA) with the proper amount of time to concentrate the whole inoculum into the final volume of 0.4~0.5 ml.

Concentrated vesicles were size-fractionated by density gradient centrifugation using the Optiprep™ medium. Vesicle sample was mixed with Optiprep™ (Sigma) 60% to make 1ml of 40% Optiprep™-vesicle mixture. Each concentration of Optiprep™ solutions (30%, 25%, 15%, 10%) was prepared diluted in deionized water and slowly pipetted into the tubes containing each sample so that the Optiprep™ gradient were visible to stack up from highest concentration to lowest concentration from bottom to up in the ultracentrifuge tube. The tube was hooked on SW 41 swinging rotor and rotated at 100,000 x g (24,149 RPM) for 16 hours at 4°C using Optima™ L-100XP Ultracentrifuge(BACKMAN COULTER, USA). Each concentration was carefully collected, 500μl from the top. The proper concentration sample was selected by silver staining and diluted > 6 fold in DPBS and centrifuged in 100 Ti rotor with a 6ml sealing tube for 8 hours at 50,000 RPM(200480 X g) using Optima™ L-100XP Ultracentrifuge(BECKMAN COULTER, USA) at 4°C in order to remove Optiprep™. Vesicle pellet was collected in the 1.5ml tube.

2.6 Silver staining of fractionated vesicle by OptiPrep™ density gradient

The proper concentration of the Optiprep™ which contains a higher concentration of vesicles with relatively less concentration of impurities has been determined using Silver staining. An aliquot of the concentration from 15% to 30% was linearized using Protein dye. 40% have been excluded because it is known to contain impurities such as free proteins, flagella, and DNAs. The samples of different Optiprep™ concentrations were loaded on 10% Mini-PROTEAN® TGX™ precast protein gel (BIO-

RAD, USA) then ran for the SDS-PAGE on PowerPac^{TM} HC(BIO-RAD, USA) with 80V. An hour and a half later, the gel was cast off and washed with pure water. Silver staining is done by following the protocol in the Silver staining kit (ProteoSilverTM, SIGMA-ALDRICH, USA).

2.7 Membrane vesicle Quantification

E.coli BW25113 wild-type and NlpI knock-out was cultured on 5ml of LB in 15ml Falcon tube and *E.coli* BW25113 ΔNlpI pCMV mCherry was cultured on 5ml of LB with 50ug/ml Kanamycin for overnight at 37°C then seed culture was diluted to OD1 and inoculated on the 500ml of LB in 1L flask for 1000 fold (for ΔNlpI pCMV mCherry strain, 50ug/ml of Kanamycin was also used). The inoculum was cultured for 24hrs at 37°C. Vesicles were purified following the 2.5 Membrane vesicle isolation. The protein concentration of each sample was measured using Qubit system including Qubit 3 Fluorometer and Qubit^{TM} Protein Assay Kit(Invitrogen, USA) with the detection range from 0.25 – 5 μg. Standard was measured on the day of measurement for calibration.

2.8 Quantification of DNA inside the membrane vesicle

Four aliquots of one vesicle sample were taken. The first sample is not treated with any chemicals and stored in 4°C. The second is treated with 0.126% Triton^{TM} X-100 (SIGMA Life Science, USA) for 30 minutes or more. The third is treated with 2.5unit/ml of DNase with 0.5mM MgCl for 30minutes in 37°C. The fourth is treated with DNase in the same way and then DNase has been deactivated by heat treatment on 56°C for 15 minutes. DNA inside the vesicle was measured using Qubit system including Qubit 3 Fluorometer and QubitTM dsDNA BR Assay Kit with a detection range from 2 – 1000ng(Invitrogen, USA). Standard was measured on the day of measurement for calibration.

2.9 Imaging the Vesicle Invasion

10,000 cell/well of Hela cell were plated on 8 well chamber and incubated at 37°C with 5% CO_2 for 36 hours. Vesicles purified from *E.coli* BW25113 ΔNlpI and *E.coli* BW25113 ΔNlpI INV/LLO was diluted in serum-free-cell culture media, MEM with Cy5 dye(Thermo Fisher Scientific, USA) which is red fluorescence dye. Cells were treated with CellTracker Violet BMQC (Thermo Fisher Scientific, USA) which green fluorescence dye. Vesicles with dye mixture were washed with DPBS 2 to 3 times and treated to the cell. The vesicle treated cells were incubated at 37°C with 5% CO_2 for 2 hours. Then cells were fixed with 5% paraformaldehyde. For the confocal settings, Cy5 set as Alexa Fluor 647, since it shares the excitation range. (633nm or 647nm)

2.10 Transfection of mammalian cells with mCherry containing vesicle

10,000 cell/well of Hela and HEK293 cells were plated on 96 well cell culture plate (SPL LIFE SCIENCES, Republic of Korea) and incubated at 37°C with 5% CO_2 for 36 hours. Mammalian cells were treated with the vesicles from *E.coli* BW25113 Δ*nlpI* INV/LLO pCMV mCherry plasmid with mammalian promotor. After 24 hours of treatment, vesicles were removed and the media was changed to serum containing media. After another 48 hours of incubation at 37°C with 5% CO_2, mCherry expressions were observed under confocal microscope.

2.11 MTT assay

50,000 cell/well of Hela cell were plated on four 12 well cell culture plate(SPL LIFE SCIENCES, Republic of Korea) and incubated at 37°C with 5% CO_2 for 36 hours. One plate is used for the whole bacteria treatment and others are used for the vesicle treatment. *E.coli* BW25113 wild-type, *E.coli* BW25113 Δ*nlpI*, *E.coli* BW25113 Δ*nlpI* INV/LLO, and *E.coli* BW25113 Δ*nlpI* INV/LLO pCMV mCherry were cultured in 5ml LB in 15ml tube overnight and diluted into 5ml LB by 100 fold 2 to 3 hours prior to the treatment. These inoculums were used as a whole bacterium and 1μl and 0.1μl of it is diluted in 1ml of MEM-serum free media. 100μl of the bacteria mix was added to each well. Bacterial cells were removed after 6 hours of incubation. MEM+Gentamycin(50μg/ml) media were added to eliminate remaining bacteria. Vesicles were purified from *E. coli* BW25113 Δ*nlpI*, *E. coli* BW25113 Δ*nlpI* INV/LLO, and *E. coli* BW25113 Δ*nlpI* INV/LLO pCMV mCherry according to the protocol above. Vesicle samples were diluted in MEM-serum free media to make a final concentration of 25μg/ml, 50μg/ml, and 100μg/ml and treated 500μl for each well and incubated at 37°C with 5% CO_2 for 12 hours. Media was removed and changed with 500μl of 10% dilution of MTT(5mg/ml) in MEM(total MMT concentration: 500μg/ml). The cells with MTT were incubated in dark at 37°C with 5% CO_2 for 2 to 4 hours until the MTT changes color. Upon removal of the 10% MTT+MEM solution, DMSO was added to the samples including empty well without MTT treatment and incubated at 37°C for 30 minutes. The 550nm absorbance was measured with Infinite M200 microplate reader(Tecan, Switzerland) using Magellan 6

2.12 Live/Dead Staining

10,000 cell/well of Hela cell were plated on three 96 well cell culture plate(SPL LIFE SCIENCES, Republic of Korea) and incubated at 37°C with 5% CO_2 for 36 hours. One plate is used for the whole bacteria treatment and others are used for the vesicle treatment. *E.coli* BW25113 wild-type, *E.coli* BW25113 Δ*nlpI*, *E.coli* BW25113 Δ*nlpI* INV/LLO, and *E.coli* BW25113 Δ*nlpI* INV/LLO pCMV mCherry were cultured in 5ml LB in 15ml tube overnight and diluted into 5ml LB by 100 fold 2 to 3

hours prior to the treatment. These inoculums were used as a whole bacterium and 1μl and 0.1μl of it is diluted in 1ml of MEM-serum free media. 100μl of the bacteria mix was added to each well. Bacterial cells were removed after 6 hours of incubation. MEM+Gentamycin(50μg/ml) media were added to eliminate remaining bacteria. Vesicles were purified from *E. coli* BW25113 Δ*nlpI*, *E. coli* BW25113 Δ*nlpI* INV/LLO, and *E. coli* BW25113 Δ*nlpI* INV/LLO pCMV mCherry according to the protocol above. Vesicle samples were diluted in MEM-serum free media to make a final concentration of 10μg/ml, 25μg/ml, 50μg/ml, and 100μg/ml and treated 100μl for each well and incubated at 37°C with 5% CO_2 for 24 hours. Media from cells were removed and cells are treated with 100μl of Calcein AM with propidium iodide solution and incubated for 10 minutes. After incubation, cells were washed with DPBS twice and treated with 5% paraformaldehyde. Dyed cells were views under a confocal microscope.

2.13 Enzyme-linked immunosorbent assay(ELISA)

50,000 Hela cells were plated on 12 well plate and incubated at 37°C with 5% CO_2 for 36 hours. One plate is used for the whole bacteria treatment and others are used for the vesicle treatment. These inoculums were used as a whole bacterium and diluted into proper concentration in which cell toxicity is low in MEM-serum free media. Bacterial cells were removed after 6 hours of incubation. MEM+Gentamycin(50μg/ml) media were added to eliminate remaining bacteria. Vesicles were purified from *E. coli* BW25113 Δ*nlpI*, *E. coli* BW25113 Δ*nlpI* INV/LLO, and *E. coli* BW25113 Δ*nlpI* INV/LLO pCMV mCherry according to the protocol above. Vesicle samples were diluted in MEM-serum free media to make proper final concentration with low cell toxicity. Media from each well is collected into a 1.5ml tube and centrifuged for 5 minutes at max speed (13,200 RPM, 16,100 X g) using centrifuge 5415R(Eppendorf, USA). Supernatants were collected and stored in -20°C. Enzyme-linked immunosorbent assay was done according to the protocol in Quantikine® ELISA Human IL-8/CXCL8 Immunoassay kit (R&D Systems, USA).

Chapter 3 Results

3.1 NlpI knock-out E. coli overproduces Membrane Vesicles

E. coli BW25113 wild-type, *E. coli* BW25113 Δ*nlpI*, and *E. coli* BW25113 Δ*nlpI* INV/LLO strains was cultured to compare the vesicle production. Produced vesicles were centrifuged and filtered to remove the bacterial cell and concentrated, then purified using different density gradient of Optiprep™ to size-fractionate the vesicles from all the impurity. (Figure 3.1.1) The proper fraction was selected according to the Silver staining results (Figure 3.1.2). The vesicle production was determined by measuring the protein concentration of the concentrated and purified vesicles. The final vesicle concentration was divided by total volume and CFU data of each sample to normalize the volume difference and total cell difference. The normalized vesicle concentration of *E. coli* BW25113 Δ*nlpI* and *E. coli* BW25113 Δ*nlpI* INV/LLO was divided by that of *E. coli* BW25113 wild-type to show the relative increment in membrane vesicle production. The fold difference between wild-type(w.t.) and NlpI knockout(Δ*nlpI*) was 60.9(±23.3)(n=3). The fold difference between wild type and Δ*nlpI* INV/LLO strain vesicle was 75.6(±27.5)($p<0.05$)(n=3). The result shows that NlpI knock-out with INV/LLO strain produces the significantly higher amount of vesicle compared to the wild type.(Figure 3.1.3)

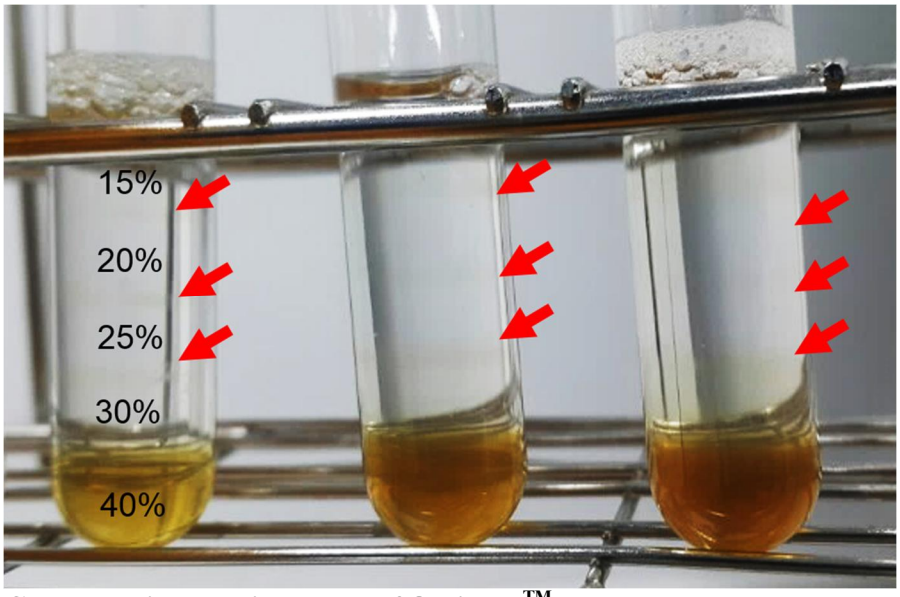

Figure 3.1.1 Concentration gradient layer of Optiprep™

Each bacterial strains were inoculated in 5ml LB in a 15ml tube with proper antibiotics for overnight. Seed cultures were inoculated into 500ml of LB in 1L flask with 1/1000-fold dilution and cultured for 24hrs. Bacterial cells were removed by centrifugation and filtration with a 0.22μ filter. Membrane vesicles were concentrated using 100KDa centrifugal filter. Concentrated membrane vesicles were placed in tube then different concentration of Optiprep™ was stacked upon sample gently so that gradient layers weren't mixed

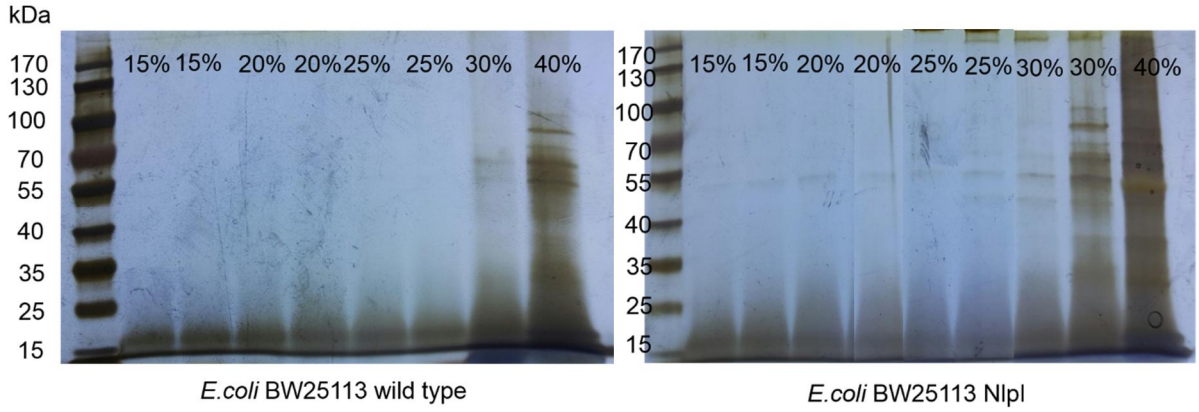

Figure 3.1.2 Silver Staining of size-fractionated Vesicle using Optiprep Density Gradient

0.5ml of each fragment of Optiprep concentration were taken from top-down then ran on the SDS-PAGE for Silver staining to show which fragment of concentration has impurity or vesicle.

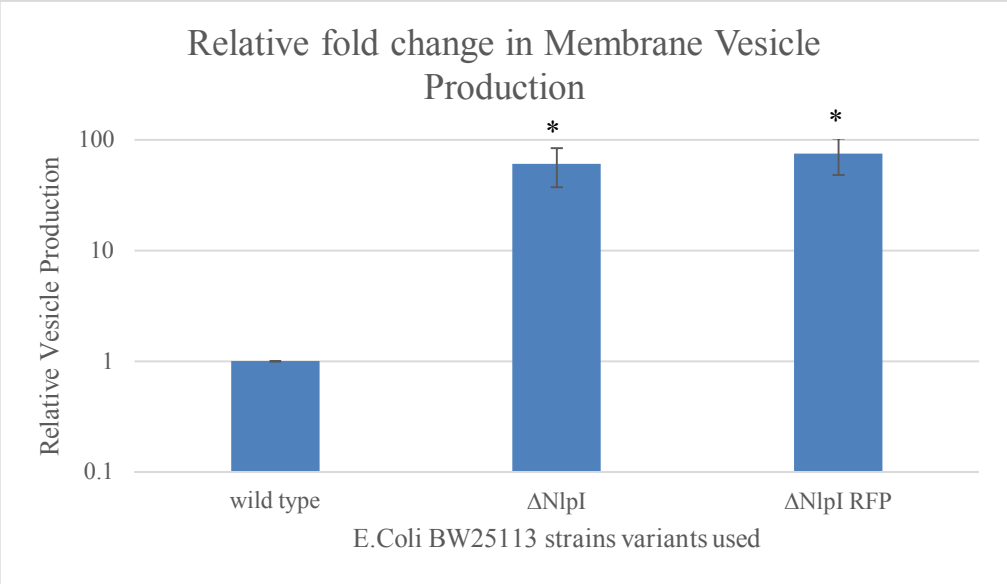

Figure 3.1.3 Relative Membrane Vesicle production

E. coli BW25113 w.t. was cultured on 5ml of LB in 15ml Falcon tube and *E.coli* BW25113 Δ*nlpI* pCMV mCherry was cultured on 5ml of LB with 50ug/ml Kanamycin for overnight at 30°C then seed culture was diluted to OD_{600} 1 and inoculated on the 500ml of LB in 1L flask with 1000 fold dilution. (for Δ*nlpI* pCMV mCherry strain, 50ug/ml of Kanamycin was also used) The inoculum was cultured for 24 hours at 30°C. The outer membrane vesicle from each sample was extracted using the centrifugal method. The cell pellets were removed by centrifuging at 7,000RPM(2739 X g) for 15 minutes using Supra 22K(HANIL, Republic of Korea) The supernatant was filtered through either Millex-GP 0.22μ syringe filter(Merck, USA) or Steritop-GP 0.22μ filter(Merck, USA) then filtered through 100kDa Amicon Ultra-15 Centrifugal Filter Units(Merck, USA) to be concentrated. The concentrated samples were purified through OptiPrep™ gradient method. 1.2 μl of w.t. vesicles and 2.4 μl of Δ*nlpI* pCMV mCherry vesicles were mixed with OptiPrep™ (Sigma, USA) to 40%(v/v) in clear ultracentrifuge tube (BECKMAN COULTER). 30%, 25%, 20%, 15% of OptiPrep™ solution diluted in deionized water were slowly pipetted into the tubes containing each sample so that the OptiPrep™ gradient were visible. The samples were centrifuged using SW41 rotor at 100000g(RCF), 4°C, for 16 hours. The centrifuged samples were collected from the top, 500μl each. The protein concentration of each sample was measured using Qubit™ Protein Assay Kit (Invitrogen, USA) and Qubit 3 Fluorometer (Invitrogen, USA) system.

3.2 *E. coli* Membrane can invade into and transfect the mammalian cell

By comparing the confocal image of Δ*nlpI* vesicle dyed with Cy5 with the negative control, the vesicles produced by *E. coli* BW25113 NlpI knock-out strain cannot invade into the Hela cell. The confocal image of vesicle treated cell shows that the vesicle with the invasin and the listeriolysin are able to invade into the human cell. (Figure 3.2.1) The 3D image was acquired using Z-stack. One intersection of the serial Z-stack image shows that the vesicles are inside of the mammalian cell rather than simply attaching to the surface. (Figure 3.2.2) The result indicates that the vesicle produced by INV/LLO plasmid containing *E. coli* BW25113 strain is essential for invasion of the vesicle into the target cell. Therefore *E. coli* BW25113 pCMV mCherry INV/LLO strain was selected to produce vesicles for the transfection into the human cell line *in vitro*.

Transfection using bacterial membrane vesicle containing pCMV mCherry plasmid was done as described on 2.10 Transfection of the mammalian cell with mCherry containing vesicle. High expression level of mCherry in both Hela and HEK 293 cells was observed. (Figure 3.2.4)

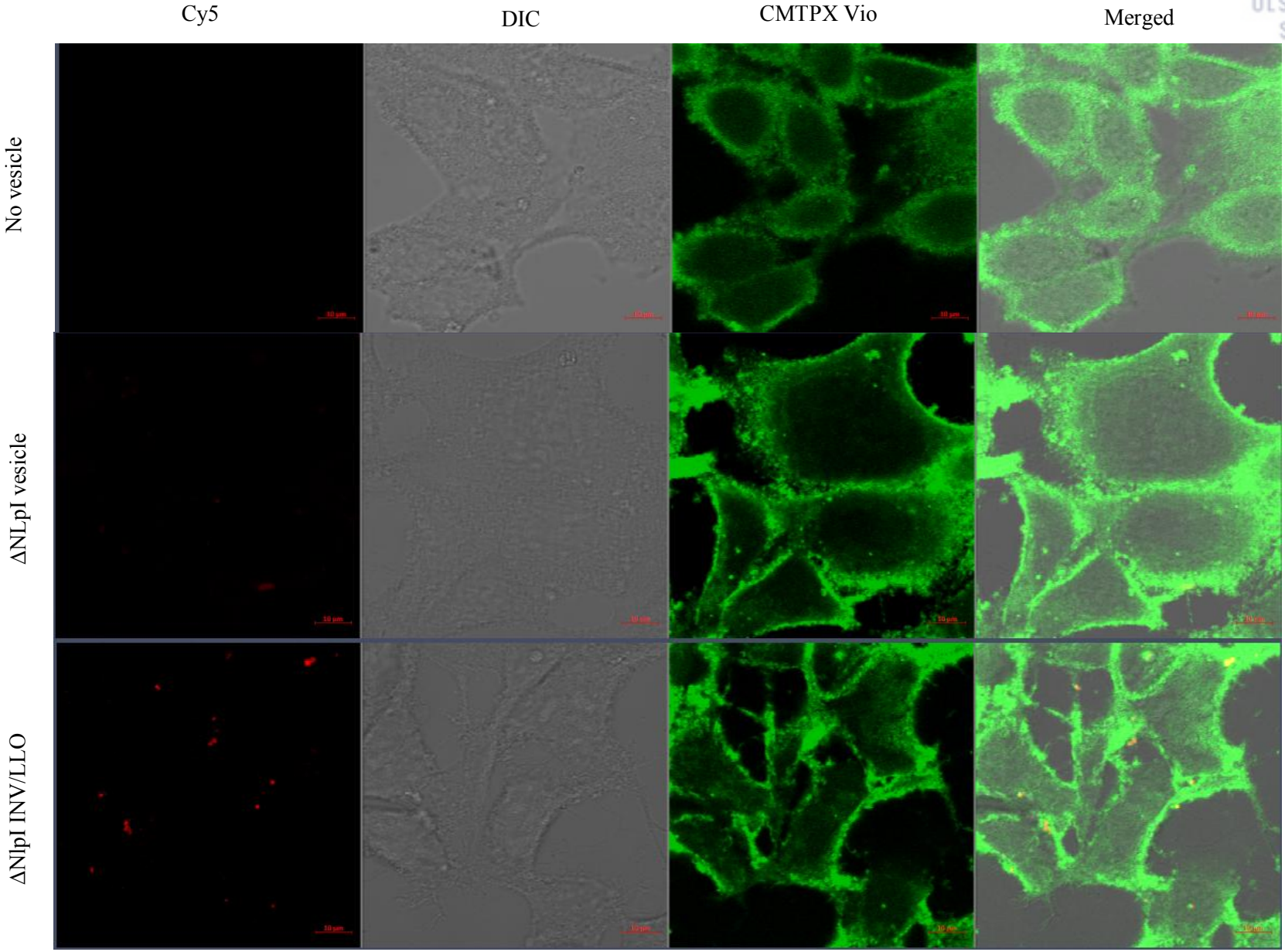

Figure 3.2.1 Confocal Image of MV invasion into Mammalian cell

Cy5 is a red-fluorescent dye with excitation and emission range of 647/665 and is treated to the vesicle. CMTPX vio is a green-fluorescent dye with excitation and emission range of 577/602 and is treated to the mammalian cell. Differential interference contrast(DIC) shows cell image.

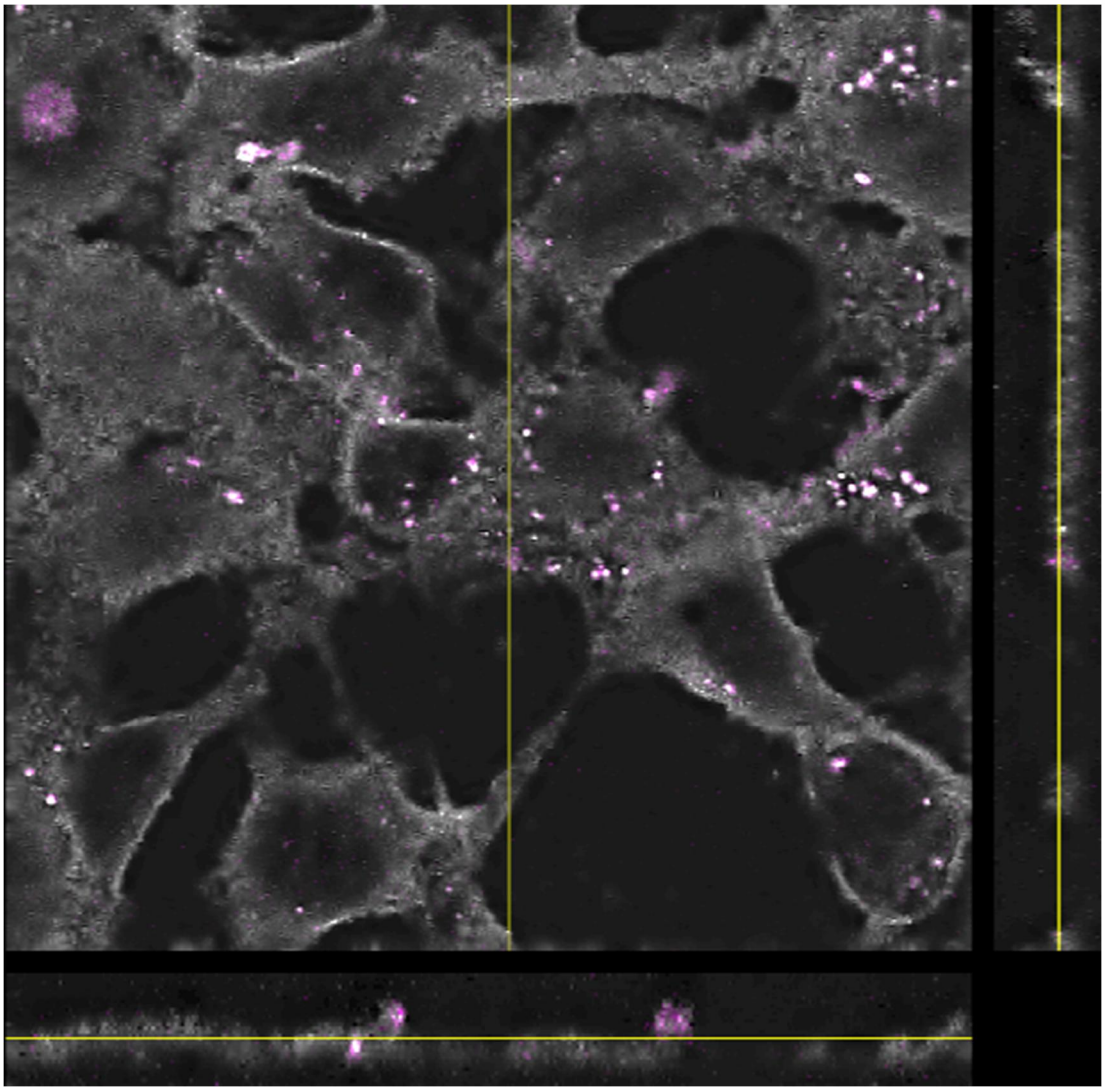

Figure 3.2.2 Dissection of Confocal Z-stack image

Showing that the vesicles are internalized in the cell. Magenta color is the vesicle/Cy5

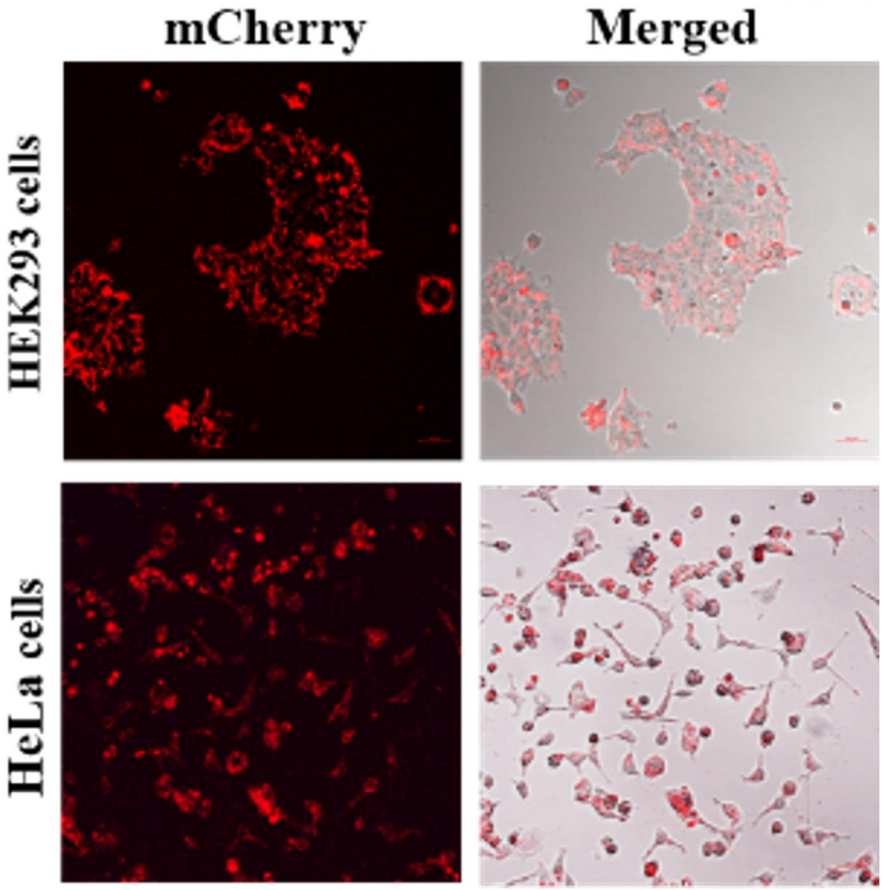

Figure 3.2.4 Confocal image of the mammalian cell with the mCherry expressions

Mammalian cells were treated with the vesicles of *E. coli* BW25113 INV/LLO Δ*nlpI* pCMV mCherry plasmid with mammalian promotor. After 24 hours, the mCherry expressions were observed under confocal microscope.

3.3 *E. coli* Membrane Vesicle are less cytotoxic towards mammalian cell lines

Whole bacteria cells of *E. coli* BW25113 wild-type and *E. coli* BW25113 Δ*nlpI* was diluted 1μl in 1ml serum-free MEM. The colony forming units(CFU) of each bacterial samples were counted to determine the multiplicity of infection (MOI) 12.4(±1.40) for *E. coli* BW25113 wild-type and MOI 19.4(±7.10) for *E. coli* BW25113 Δ*nlpI*. Before measurement, the plates were shaken for 10 seconds with 2.5 magnitudes, orbitally to ensure even distribution of MTT dye. The MTT absorbance of 550nm from each well was taken from 3 X 3 spot. MTT absorbance of 550nm of each sample was divided with that of the negative control which is the Hela cell without any treatment to calculate relative viability(%) of each sample. The MTT assay result shows that vesicle produced are relatively non-cytotoxic on the mammalian cell with the concentration lower than 25μg/ml compared to the whole bacteria treatment. (Figure 3.3.1 and Figure 3.3.2) 100μg/ml of *E. coli* BW25113 Δ*nlpI* vesicle showed 76.3(±13.5)% of relative viability which is compatible to the relative viability of whole bacteria treated samples such as relative viability of 76.8(±18.6)% of *E. coli* BW25113 wild-type bacteria treated sample or 76.3(±14.0)% of *E. coli* BW25113 Δ*nlpI* whole bacteria treated one.(Figure 3.3.2) The t-test revealed that it is significantly different from the negative control(*$p<0.05$). *E. coli* BW25113 Δ*nlpI* INV/LLO vesicle with 100μg/ml concentration showed 83.2(±4.74)% of relative viability with a significant difference compared to the negative control(**$p<0.01$). *E. coli* BW25113 Δ*nlpI* INV/LLO vesicle with 50μg/ml is also showed significant difference with the control(*$p<0.05$). (Figure 3.3.1)

The confocal image reveals the morphology of the vesicle treated cell is in healthy shape and no morphology change. (Figure 3.3.3)

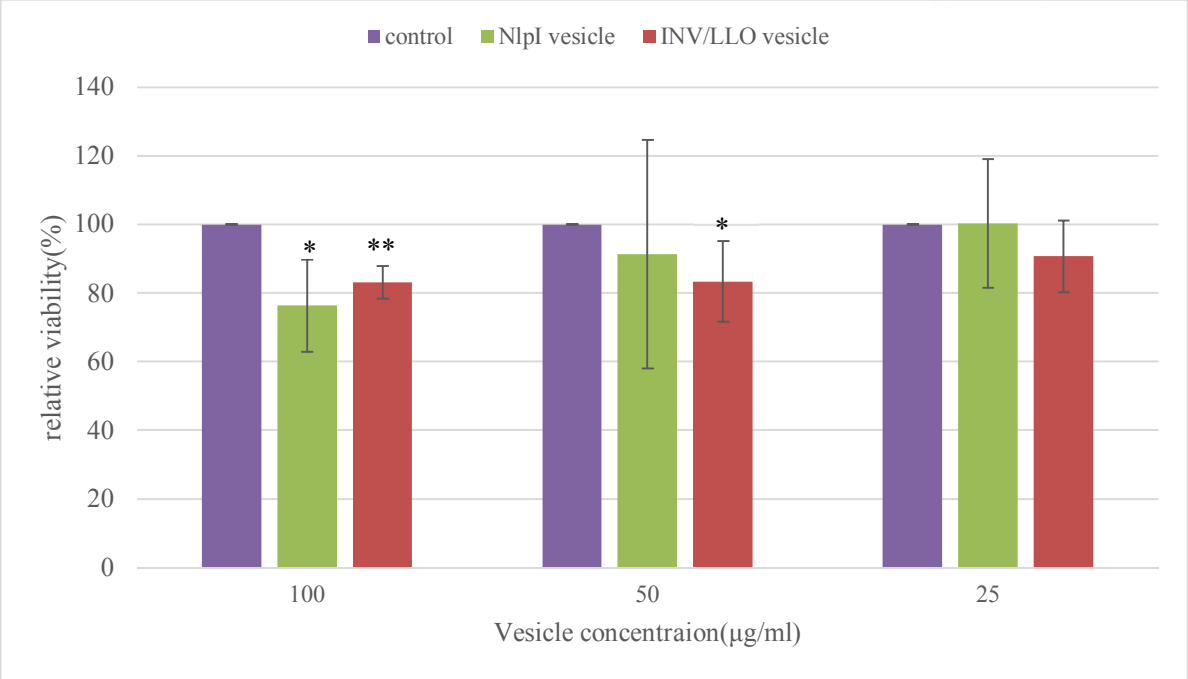

Figure 3.3.1 MTT assay result of Vesicle treated samples
Relative viability of each vesicle treated Hela cell samples compared with negative control which is the Hela cell without any treatment. *p<0.05, **p<0.01

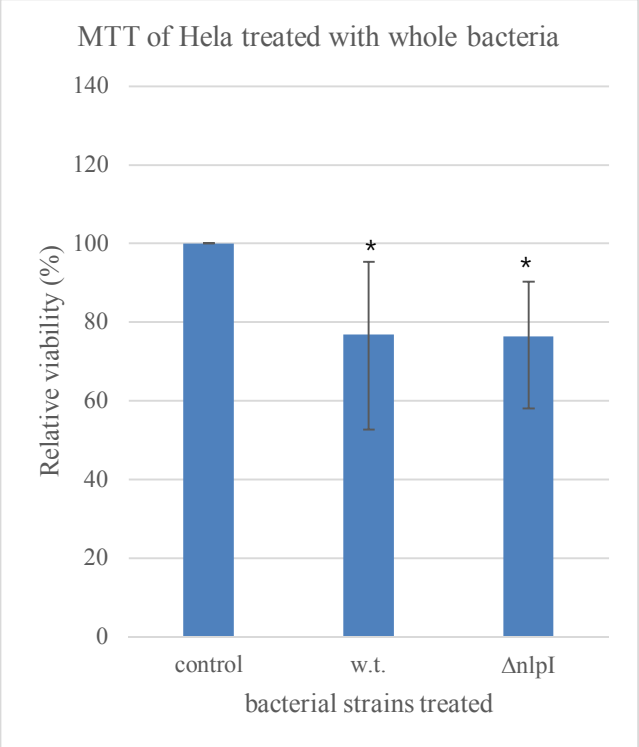

Figure 3.3.2 MTT assay result of the whole cell treated

Each Hela MTT sample is compared with the negative control group and divided by negative control value to show the relative viability. *p<0.05

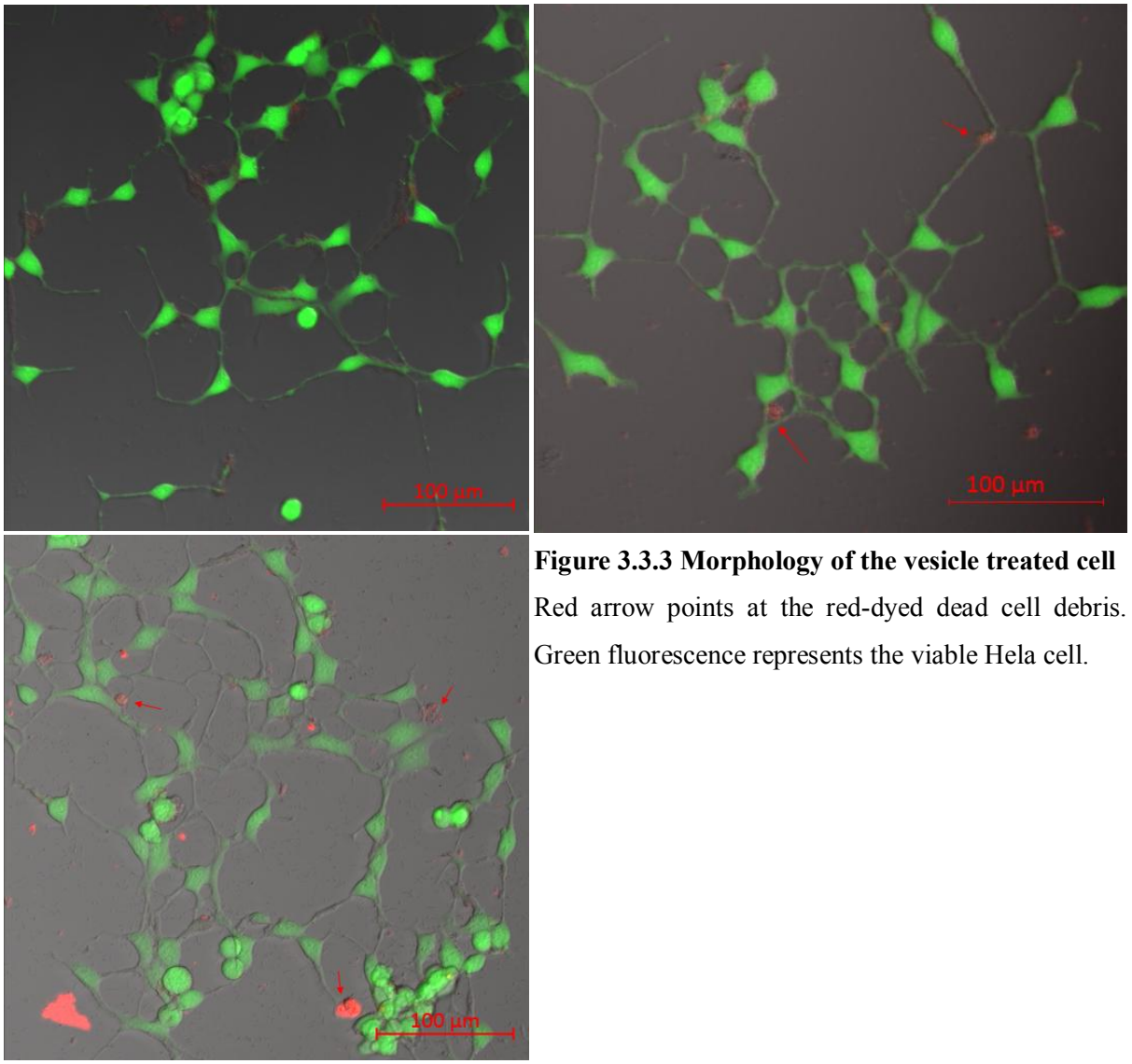

Figure 3.3.3 Morphology of the vesicle treated cell

Red arrow points at the red-dyed dead cell debris. Green fluorescence represents the viable Hela cell.

Chapter 4 Conclusion

The current advance in the biology and biotechnology field aims at individualized treatment, especially genetic manipulation for gene therapy. However, a proper transfecting agent that is applicable both *in vivo* and *in-vitro* with easy application, massive production, and low risk is scarce[5, 14, 15, 19, 21, 23]. Therefore, through studies, I suggest bacterial membrane vesicle as a possible candidate for the delivery vector.

By transforming *E. coli* type strains with the desired plasmid which is associated with cell invasion, invasion into the human cell was significantly increased so that was visualized in the confocal image. Using the *nlpI* knock-out strain to produce a large quantity of vesicle compare to wild-type not only secures the amount of vesicle required for the study but also suggest that the bacterial membrane vesicle can be produced in large quantity which is essential when it comes to commercialization.

The invasion of bacterial MVs into the mammalian cells were demonstrated and the transfer of the plasmid into the mammalian cell and its expression was verified.

The cytotoxicity assay such as MTT assay was done to determine the maximum concentration of the bacterial membrane vesicle that does not exert the cell death nor morphology change. Comparing the viability of the mammalian cell treated with different concentrations of the bacterial membrane vesicle with those of the Lipofectamine 2000 which is a commercialized and widely used chemical transfecting agent revealed that the bacterial membrane vesicle is far less cytotoxic than the chemical transfecting agent. The non-cytotoxic concentration of the bacterial membrane vesicle was 25μg/ml whereas the Lipofectamine 2000 treatment showed 25% cell viability with 20μg/ml.[40]

Through my study, I have demonstrated that the bacterial vesicle can be mass produced, invades into the target cell and delivers the DNA without killing or changing the target cell, therefore denoting the potential of the bacterial membrane vesicles as a candidate for a new omni-transfectable transfecting agent.

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